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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/36, 37/26, 37/02

A2

(11) International Publication Number: WO 94/16723

(43) International Publication Date: 4 August 1994 (04.08.94)

(21) International Application Number: PCT/US94/00799

(22) International Filing Date: 25 January 1994 (25.01.94)

(30) Priority Data:
08/011,031 29 January 1993 (29.01.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/011,031 (CIP)
Filed on 29 January 1993 (29.01.93)

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: WOUND HEALING COMPOSITION

(57) Abstract

Pharmaceutical wound healing compositions are provided which comprise insulin-like growth factor-1 in combination with insulin-like growth factor binding protein. The pharmaceutical compositions are formulated for non-systemic administration. The present invention also provides methods of using such wound healing compositions.

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# WOUND HEALING COMPOSITION

## Field of the Invention

This invention relates to pharmaceutical compositions for wound healing comprising the combination of insulin-like growth factor and insulin-like growth factor binding protein and to methods of using the compositions.

## Background of the Invention

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2, respectively) are seven kDa proteins that are related in structure to each other and to insulin. IGF-1 and IGF-2 are growth and differentiation factors for most cells in the body and are present at high concentrations in serum (about 300 ng/ml for IGF-1 and 1000 ng/ml for IGF-2). Circulating levels of IGF-1 are determined primarily by growth hormone, which stimulates the liver to make IGF-1. Most of the growth-promoting effects of growth hormone are believed to be mediated by IGF-1. The combination of growth hormone and IGF-1 is shown to stimulate linear growth and weight gain as described in PCT Patent Application No. US91/03841.

The use of various growth factors to enhance wound healing is suggested in J. Van Brunt and A. Klausner, BioTechnology, 6:25-30, 1988. IGF-2 was shown to be an effective treatment for wounds, as reported in U.S. Patent 4,885,163 to Sharr and Smith. IGF-1 alone, however, was not effective in partial thickness wounds, but acted synergistically in combination with PDGF (platelet derived growth factor) to promote connective tissue and epithelial growth, as described in Lynch, et al., J. Clin. Invest. 84:640-646, 1989 and Van Brunt and Klausner, Bio/Technology 6:25-30, 1988. U.S. Patent 4,983,581 reports the use of IGF-1 and TGF-$\beta$ in a wound healing composition. PCT Patent Application Publication No. WO91/18621 describes the use of IGF-1 in combination with growth hormone to enhance growth and weight gain, but not for wound healing.

IGF-1 and IGF-2 circulate in blood bound to specific binding proteins of which six are now known (IGFBP-1 to IGFBP-6). The binding proteins bind 95% or more of the IGFs in blood. When bound by binding proteins, IGF-1 and IGF-2 are prevented from

interacting with cell surface receptors which mediate their biological functions. Insulin like growth factor binding protein-1 (IGFBP-1, or BP-1) is a 23 kDa IGF binding protein. A published in vivo experiment that has been performed with IGFBP-1 indicates that IGFBP-1 acts as an IGF-1 inhibitor in vivo. Lewitty et al., Endocrinology, 129:2254-2256, (1991) found that IGFBP-1 inhibited the hypoglycemic response of rats given intravenous infusions of IGF-1, which promotes glucose uptake by cells. This property can lead to hypoglycemia when IGF-1 is present in sufficient amounts. Publications by other groups indicate that IGFBP-1 is expressed in vivo during periods of growth arrest (e.g., starvation and diabetes), also suggesting that IGFBP-1 might act as an IGF-1 inhibitor.

In vitro experiments have yielded contradictory conclusions as to whether IGFBP-1 potentiates or inhibits the effects of IGF-1 on cells and tissues. IGFBP-1 was reported to inhibit the effects of IGF-1 on cultured endometrial cells (Rutanen et al., J. Clin. Endocrinol. Metab. 60:173-180, 1988), choriocarcinoma cells (Ritvos et al., Endocrinology, 122:2150-2157, 1988), thyroid follicular cells (Frauman et al., Endocrinology, 124:2289-2296, 1989), fibroblasts (Liu et al., Biochem. Biophys. Res. Comm., 174:673-679, 1991) and chicken cartilage cells (Burch et al., J. Clin. Endocrinol. Metab., 70:173-180, 1990). In contrast, Elgin et al., Proc. Natl. Acad. Sci. USA, 84:3254-3258, (1987) reported that IGFBP-1 potentiated the mitogenic effects of IGF-1 on fibroblasts and smooth muscle cells in the presence of platelet poor plasma. Subsequent studies (Clemmons and Gardner, Journal of Cellular Physiology, 145:129-135, 1990) showed that an additional factor in platelet poor plasma was required for IGFBP-1 to potentiate the effects of IGF-1 on these cells. The identity of this factor is not known. Koistinen et al., Biochem. Biophys. Res. Comm., 173:408-415, (1990) reported that IGFBP-1 inhibited the binding of IGF-1 to human fibroblasts but paradoxically stimulated their DNA synthesis.

The use of the combination of IGF-1 and IGFBP-3 for anabolism is described in PCT patent application publication number WO92/13556. Systemic administration of IGFBP-3 was found to potentiate the anabolic effects of IGF-1, whereas another

study found systemic administration of IGFBP-1 inhibited the anabolic effects of IGF-1.

Since wound healing is mediated by a myriad of components, another wound healing composition which contains natural human proteins and which is easy to administer and manufacture would be a beneficial contribution to the existing pharmacopeia available for wound healing. The present invention provides a novel composition for wound healing.

Summary of the Invention

The present invention describes the use of a combination of IGF-1 and IGFBP, particularly IGFBP-1, in a composition for non-systemic administration for wound healing. Since it has been known that IGFBP-1 inhibits the activity of IGF-1 systemically, it is indeed surprising to discover that IGFBP-1 potentiates IGF-1 when used in non-systemic administration for wound healing.

The present inventors have determined that IGFBP-1 acts as an IGF-1 inhibitor in vitro and when given systemically in vivo. In contrast, they have found that IGFBP-1 potentiates the growth-promoting effects of IGF-1 when applied non-systemically for wound healing. The potentiating effect of IGFBP-1 in non-systemic healing may be due to the ability of IGFBP-1 to prolong the half-life of IGF-1 at a wound site, the ability of IGFBP-1 to protect IGF-1 from extracellular proteases in the wound, or a true synergistic interaction of the two factors.

This invention is directed to a composition comprising IGF-1 and IGFBP and a pharmaceutically acceptable carrier suitable for non-systemic administration for use in wound healing.

In addition, this invention is directed to a method for promoting the rate or improving the quality of wound healing, which comprises non-systemically administering to such wound IGF-1 and IGFBP in a therapeutically effective amount to promote wound healing.

Detailed Description of the Invention

The present invention relates to pharmaceutical compositions comprising IGF-1, IGFBP and a pharmaceutically acceptable carrier suitable for non-systemic administration for use in wound healing. Although the invention is described with respect to IGF

binding protein 1 (IGFBP-1 or BP-1), the present inventors contemplate the use of any of the six known IGF binding proteins.

Terms used throughout this specification are defined as follows:

The term "IGF" refers to any polypeptide that binds to the IGF type I Receptor, including, for example, IGF-1, IGF-2, (des1-3)IGF-1, met-IGF-1, insulin, and any active fragments which bind to the type I Receptor. This hormone family is described in Blundell and Humbel, Nature, 287:781-787 (1980). Due to this common receptor binding, the teachings of the present invention which are described with respect to IGF-1 are intended to encompass IGF-2, (des1-3)IGF-1, met-IGF-1, insulin, and any active fragments which bind to the type I Receptor.

The term "IGF-1" refers to natural human IGF-1, recombinantly produced human IGF-1, met-IGF-1, and any active fragments of IGF-1.

The term "IGFBP" refers to any of the six known IGF binding proteins or to active fragments of these binding proteins which bind to IGF.

The term "pharmaceutically acceptable carrier" refers to a physiologically-compatible, aqueous or non-aqueous solvent.

The term "patient" refers to any human or animal in need of treatment for wound healing.

The term "non-systemic" refers to any route of administration which does not directly involve the use of blood or blood vessels. Examples of formulations useful for non-systemic administration include salves, ointments, creams, gels, lotions, aerosols, powders, liquids or solids. Non-systemic administration includes topical routes, intradermal injections, suppositories, enema, inhaled aerosol, oral routes, and any non-circulatory route of administration.

The compositions of the present invention comprise IGF-1 and IGFBP, particularly IGFBP-1, in a pharmaceutically acceptable carrier. IGF-1 can be obtained commercially from Bachem in Torrance, California. IGF-1 can also be purified from natural sources, for example from serum. IGF-1 can also be prepared by methods well known to those skilled in the art including, for example, recombinant techniques such as those set forth in the

examples below. Although the expression system of the recombinant methods described in the examples employs a particular bacterium, it is contemplated that yeast, bacterial, mammalian, insect or other expression systems can also be used. IGF-1 can also be synthesized using conventional methods known in the art

IGFBP-1 can be purified from natural sources such as amniotic fluid, or can be produced in accordance with procedures described in PCT Application publication WO 89/09792, published on October 19, 1989, incorporated herein by reference. IGFBP-1 can also be produced by employing the procedures used by the inventors of the present application, as set forth in the examples below. The present inventors have performed in vitro and in vivo experiments to determine the interaction of recombinant IGFBP-1 with IGF-1.

Compositions containing a molar ratio of IGF-1 to IGFBP-1 between 1:100 and 100:1 are contemplated. It is believed compositions containing concentrations of IGF-1 of less than 0.01 $\mu g/25\ mm^2$ of surface area of wound may not be effective while compositions containing concentrations of IGF-1 of more than 500 $\mu g/25\ mm^2$ of surface area of wound may have undesirable side effects, such as elevated levels of circulating IGF-1. The frequency of dosing will depend on pharmacokinetic parameters of the IGF-1 and IGFBP-1 in the formulation used and can be readily determined by those skilled in the art.

The formulations of this invention are designed for non-systemic administration. One formulation incorporates IGF-1 and IGFBP-1 into liquid form in which physiological saline solution may be used as a carrier. It is contemplated that other pharmaceutically acceptable carriers may also be used. The liquid formulations comprise protein and a carrier, such as phosphate buffered saline (PBS). The liquid form may be applied directly to the wound, injected intradermally, or used to saturate an occlusive dressing.

In addition to liquid form, it is also contemplated that a formulation may incorporate IGF-1 and IGFBP-1 into a salve, ointment, cream, gel, lotion, topical aerosol, or powder.

Ointments generally are prepared using either (1) an oleaginous base, i.e., one consisting of fixed oils or hydrocarbons, such as white petrolatum or mineral oil, or (2) an absorbent base, i.e., one consisting of an anhydrous substance or substances which can absorb water, for example, anhydrous lanolin. Customarily, following formation of the base, whether oleaginous or absorbent, the active ingredients (IGF-1 and IGFBP-1) are added in an amount affording the desired concentration.

Creams and lotions are oil/water emulsions. They consist of an oil phase (internal phase), comprising typically fixed oils, hydrocarbons, and the like, such as water-soluble substances, including, for example, added salts. The two phases are stabilized by use of an emulsifying agent, for example, a surface active agent, such as sodium lauryl sulfate, hydrophilic colloids, such as acacia colloidal clays, veegum, and the like. Upon formation of the emulsion, the active ingredients (e.g., IGF-1 and IGFBP-1) customarily are added in amounts to achieve the desired concentration.

Gels comprise a base selected from an oleaginous base, water, or an emulsion-suspension base, as previously described. To the base is added a gelling agent which forms a matrix in the base, increasing its viscosity. Examples of gelling agents are hydroxypropyl cellulose, acrylic acid polymers, and the like. Customarily, the active ingredients (e.g., IGF-1 and IGFBP-1) are added to the formulation at the desired concentrations prior to the addition of the gelling agent.

In one embodiment, it is envisioned that the carrier and the active ingredients are formulated in a physiologically-compatible, slow-release formulation. The primary solvent in such a formulation may be either aqueous or non-aqueous in nature. In addition, the formulation may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, odor, rate of dissolution, absorption, or release of the active ingredients. Such excipients are those substances usually and customarily employed to formulate dosages for administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile vials or containers as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. Formulations containing IGF-1 and IGFBP-1 are stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The present invention also relates to methods for treating wounds by administering the above described pharmaceutical compositions to a patient in need thereof.

The manner of administering the therapeutic compositions of the present invention containing for example, IGF-1 and IGFBP-1, can be via non-systemic methods, including topical applications, intradermal injection, suppositories, enema, inhaled aerosol, or oral routes. To achieve and maintain the desired dose of IGF-1 and IGFBP-1, repeated doses may be administered. Any of these methods are intended to create a preselected concentration range of IGF-1 and IGFBP-1. Those skilled in the art can readily determine the appropriate mode of administration and dosage depending on various factors including, for example, the type and location of the wound, the age and condition of the patient, and the formulation used. Examples of the types of wounds treatable using the compositions of the present invention are chemical or thermal burns; skin graft donor and transplant sites; cutaneous ulcers, including but not limited to decubitus ulcers, diabetic ulcers, venous stasis ulcers, and necrobiosis lipoidicum ulcers; surgical wounds, wound dehiscence, including but not limited to the abdominal, thigh, and chest areas; corneal trauma and transplants; tooth extractions and oral surgery; disruption of a mucous membrane, including but not limited to the gastrointestinal tract (ulcerative colitis) and bladder; and any of a wide range of other traumatic interruptions of connective tissue, e.g., abrasions. The pharmaceutical compositions of the present invention are particularly useful for dermal wounds.

The effects of IGF-1 and IGFBP-1 alone and in combination on wound healing were tested using the rabbit ear dermal ulcer model, as described more fully in the examples below. Briefly, IGF-1 and IGFBP-1 in varying ratios or control buffer were applied to an induced wound in the models. Tissues from the wound site were subjected to histologic analysis. IGF-1 or IGFBP-1 alone did not have a significant effect on healing compared with controls. The combination of IGF-1 with IGFBP-1 showed significantly increased wound healing compared with controls. The enhancement was greatest when IGF-1 was in molar excess.

Experiments were also conducted using homozygous db/db mice, which are diabetic and exhibit delayed wound-healing compared to normal mice or heterozygous db/+ mice. Experiments were designed to compare the effects of IGF-1 and IGFBP-1 alone and in combination on wound-healing in this model. Parameters measured were: (1) percent re-epithelialization; (2) new granulation tissue; and (3) capillary number. The combination of IGF-1 + IGFBP-1 caused a dose-dependent increase in each of these wound healing parameters. The responses to the combination of IGF-1 and IGFBP-1 were better than the responses to either protein alone.

The following examples are illustrative only and are not intended to limit the invention.

## EXAMPLE 1

### Construction of the IGF-1 gene

The IGF-1 gene was assembled in two stages. Initially, the DNA sequence encoding IGF-1 was joined to DNA sequences encoding the secretory leader sequence of the E. coli OMP A protein ($ompA_L$). This gene fusion was constructed in order to determine whether IGF-1 could be efficiently secreted from E. coli. A second construct, in which IGF-1 is expressed as an intracellular protein in E. coli, was created by deleting DNA sequences encoding the OmpA leader sequence and replacing them with DNA sequences that allow intracellular expression of IGF-1.

### Construction of the $OmpA_L$-IGF-1 gene fusion

The four synthetic oligonucleotides labeled OmpA1U (SEQ ID NO:1), OmpA2U (SEQ ID NO:2), OmpA1L (SEQ ID NO:3) and OmpA2L (SEQ

ID NO:4), were annealed pairwise (1U + 1L and 2U + 2L) and the pairs ligated together. All four of these oligonucleotides were synthesized using DNA synthesizers purchased from Applied Biosystems (Models 391 and 380A). The ligation mixture was then digested with the restriction enzyme HaeIII. The resulting BamHI/HaeIII restriction fragment coding for a translational start signal and the first 21 amino acids of the ompA signal sequence was purified. This DNA fragment was mixed with BamHI + PstI-digested PUC18 DNA (Boehringer Mannhein Biochemicals, Indianapolis, IN) and the two synthetic oligonucleotides [IGF-1 (1-14) U + L] (SEQ ID NO:5 and SEQ ID NO:6) were ligated together. The ligation mixture was used to transform E. coli strain JM109 (New England Biolabs, Beverly, MA) and individual colonies isolated. These plasmids ($OmpA_L$IGF-1pUC18) have a translational start signal followed by DNA sequences encoding the OmpA signal sequence and the first 14 amino acids of IGF-1.

An M13 phage containing DNA sequences encoding amino acids 15 through 70 of IGF-1 was created by ligating together the two complementary pairs of oligonucleotides (IGF1U + 1L and IGF2U + 2L) (SEQ ID NO:7 and SEQ ID NO:8) and cloning the DNA fragment into PstI + HindIII-digested M13 mp19 DNA (New England Biolabs, Beverly, MA). Double-stranded DNA was purified from a phage clone and the PstI/HindIII fragment encoding amino acids 15-70 of the IGF-1 protein were isolated. This DNA fragment was ligated together with PstI + HindIII-digested plasmid $OmpA_L$IGF-1pUC18 DNA and used to transform E. coli strain JM107 (GIBCO BRL, Gaithersburg, MD). The BamHI/HindIII fragment containing the IGF-1 gene fused to the $OmpA_L$ sequence was isolated and cloned into the BamHI + HindIII generated site of plasmid pT3XI-2 (described in PCT Application publication WO 91/08285 published on June 13, 1991). The completed plasmid containing the $ompA_L$-IGF-1 gene fusion is called pT3XI-2 $\phi 10_C$(TC3)$ompA_L$IGF-1.

Construction of the Methionyl-IGF-1 gene.

The BamHI/HindIII fragment containing the $OmpA_L$-IGF-1 gene fusion described above was purified from plasmid pT3XI-2$\phi 10_C$(TC3)$ompA_L$IGF-1 and digested with HinfI. The approximate 200 bp HinfI/HindIII DNA fragment was mixed with the annealed, complementary synthetic oligonucleotides (MetIGF1U + 1L) (SEQ ID

NO:9 and SEQ ID NO:10) and ligated with BamHI + HindIII-digested plasmid pT3XI2 DNA, and used to transform E. coli JM107. The completed plasmid construct is called $\phi 10_C$(TC3)IGF-1pT3XI-2 and contains an extra alanine residue in between the initiator methionine and the beginning of the IGF-1 sequence. The BamHI/HindIII fragment containing the mutant IGF-1 gene was isolated and ligated into the BamHI + HindIII generated site of plasmid pT5T (described in Nature, Vol. 343, No. 6256, pp. 341-346, 1990). The ligation mixture was used to transform E. coli BL21/DE3 described in US Patent 4,952,496 and the resulting individual colonies were isolated. This construct was named $\phi 10_C$(TC3)IGF-1pT5T.

The extra alanine codon was removed by in vitro mutagenesis. In vitro mutagenesis was performed using a Muta-Gene kit (Bio-Rad Laboratories (Richmond, CA). The mutagenesis procedure followed was essentially that described in the instructions that accompany the kit. Plasmid $\phi 10_C$(TC3)IGF-1pT3XI-2 was digested with BamHI + HindIII and the ~200 bp DNA fragment containing the mutant IGF-1 gene was purified and cloned into the BamHI and HindIII sites of plasmid M13 mp19.

Uracil-containing single-stranded template DNA was prepared following propagation of the phage in E. coli strain CJ236 (supplied with Muta-Gene Kit, Bio-Rad Laboratories, Richmond, CA). The oligonucleotide used for mutagenesis had the sequence: 5' - GATGATTAAATGGGTCCGGAGACT - 3' (SEQ ID NO 11). The mutagenesis reaction product was used to transform E. coli strain JM109 and individual plaques picked.

Double-stranded replicative form DNA from individual phages was isolated, digested with BamHI + HindIII and the ~200 bp fragment containing the IGF-1 gene purified. The purified DNA was cloned into the BamHI + HindIII generated site of plasmid pT5T and used to transform E. coli strain BL21/DE3. Several isolates were sequenced, and all were correct. One bacterial colony with the correct plasmid was named $\phi 10$(TC3)mutIGF-1pT5T.

Expression of Met-IGF-1 in bacteria

For small-scale experiments, an overnight culture of E. coli strain $\phi 10$(TC3)mutIGF-1pT5T was diluted 1:100 into 800 ml of

Luria Broth (10 g/liter tryptone, 5 g/liter yeast extract and 10 g/liter NaCl, pH 7.5) medium containing 15 μg/ml tetracycline and grown at 37° until the optical density at 600 nm was 0.7-0.9. IPTG (isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 1 mM and the culture grown for an additional 2.5-3.0 hours at 37°C. At the end of the induction period, the cells were harvested by centrifugation. The cell pellet was washed once with ice-cold buffer A (50 mM Tris-HCl pH 7.5/ 25 mM NaCl/1 mM DTT) and stored frozen at -70°C or resuspended in buffer A and used immediately.

For large-scale experiments, E. coli strain ϕ10(TC3)mutIGF-1pT5T was grown in a 10 liter fermenter at 37°C in complex media (40 g/l NZ amine HD, 2 g/l $KH_2PO_4$, 1 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l $Na_2SO_4$, 1 g/l $Na_3$ citrate $\cdot 2H_2O$, 50 g/l glycerol, 0.1 ml/l Macol 19::GE60, 2 ml/l trace minerals, 20 mg/l thiamine HCl, and 15 mg/l tetracycline HCl, pH 7). When the optical density of the culture reached approximately 10, IPTG was added to a final concentration of 0.1 mM. Bacteria were grown for an additional 2-8 hours, harvested by centrifugation and the cell pellet stored at -70°C until use.

## EXAMPLE 2

Purification of Met-IGF-1

E. coli cells were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM DTT), and were disrupted at 1800 psi using a French pressure cell. The suspension was centrifuged at 20,000 x g for 30 minutes, and aliquots of the pellet and the supernatant were analyzed by SDS-PAGE. A major band corresponding to Met-IGF-I was present in the pellet, but not the supernatant. The pellet was resuspended in Buffer A (40 ml/10 g cell paste), and re-centrifuged at 20,000 x g for 30 minutes. This wash procedure was repeated 2 times. The final pellet containing Met-IGF-I was resuspended in 6 M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT (25 ml/10 g cell paste) using a ground glass homogenizer, and the suspension was incubated at room temperature for 15 minutes. The undissolved protein was removed by centrifugation at 20,000 x g for 30 minutes. SDS-PAGE analysis of the pellet and supernatant showed that Met-IGF-I was present in the supernatant only.

Refolding of Met-IGF-1

The denatured and reduced Met-IGF-1 was subjected to a three-step refolding protocol.

1) The oxidizing agent, oxidized glutathione (GSSG) was added to the supernatant from Example 2 to a final concentration of 25 mM, and incubated at room temperature for 15 minutes.

2) The solution was then diluted 10 fold gradually with 50 mM Tris, pH 9.7 to a final concentration of 150 - 300 µg/ml. Cysteine was added to a final concentration of 5 mM to aid in disulfide exchange.

3) The solution from step (2) was incubated overnight at 4°C to allow completion of disulfide exchange, and then centrifuged at 20,000 x g for 15 minutes. SDS-PAGE analysis of the pellet and the supernatant showed that the supernatant was composed of relatively homogeneous Met-IGF-I.

Aliquots (50 µl) of the supernatant were diluted to 1 ml with Buffer C (0.05% trifluoroacetic acid)(TFA, Pierce, Rockford, IL), injected onto a reverse phase column (RP-4, 1 x 250 mm, SynChrom, Lafayette, IN), and eluted with Buffer D (80% acetonitrile in water, 0.042% TFA) using a linear gradient (increase of 1% Buffer D/minute) at a flow rate of 0.1 ml/minute.

Two major peaks were resolved: Peak I at 56.5 minutes, and Peak II at 58.2 minutes. In addition, a minor peak was present at 60 minutes, and a broad peak at 75-79 minutes containing improperly refolded Met-IGF-I species. Based on the integration of the HPLC chromatogram, Peak I and Peak II represented approximately 25% and 30% of the crude Met-IGF-I protein loaded onto the reverse phase column, respectively. N-terminal sequence analysis of Peak I and Peak II gave the sequence MetGlyProGluThrLeu... (SEQ ID NO:12), which matches the N-terminal amino acid sequence of human IGF-I except for the extra methionine residue at the N-terminus. Recombinant human Met-IGF-I (Bachem, Torrance, CA) eluted at a retention time identical to Peak II. Therefore, Peak II represents correctly refolded Met-IGF-I, as evidenced by retention time identical to the purchased standard as well as biological activity identical to the purchased standard. IGF-1 which has not been correctly refolded exhibits reduced or no biological activity.

**EXAMPLE 3**

Isolation of Correctly Refolded Met-IGF-1

The following is a description of the preparation of met-IGF-1 from 305 g of cell paste. The supernatant from the refolding procedure of Example 2 (6700 ml) was concentrated 10-fold and exhaustively dialyzed against 20 mM HEPES, pH 7.5. The dialyzed sample was centrifuged 20,000 x g for 15 minutes to remove precipitated proteins, passed through a 0.2 μm filter (Corning, Corning, NY) and loaded onto an S-Sepharose column (5.0 x 40 cm, Pharmacia LKB, Piscataway, NJ) previously equilibrated with the same buffer, at a flow rate of 40 ml/minute. The bound met-IGF-1 was eluted with a 5000 ml linear gradient to 0.5 M NaCl at a flow rate of 40 ml/minute. 25 ml fractions were collected. Two symmetrical peaks were resolved: Peak A eluting at 0.12 M NaCl, and Peak B eluting at 0.15 M NaCl. SDS-PAGE analysis of aliquots of Peaks A and B showed that they contained relatively homogeneous IGF-1 (> 90% homogeneous); however, several high molecular weight *E. coli* proteins were still present. The S-Sepharose fractions corresponding to Peaks A and B were pooled separately. HPLC analysis (RP-4, 1 x 250 mm) of the S-Sepharose pools showed that Pool A and B were composed of major peaks eluting at 56.5 minutes and 58.2 minutes, respectively, as well as several minor peaks. The major RP-4 peak of the S-Sepharose pool B eluted with the same retention time as commercially purchased recombinant human met-IGF-1 (Bachem, Torrance CA).

The S-Sepharose pool B was made to 2 M NaCl, 20 mM HEPES, pH 7.5, and loaded at a flow rate of 30 ml/minute onto a Toyopearl Butyl-650S 5.0 x 25 cm( Supelco, Bellefonte, PA) hydrophobic interaction column previously equilibrated with 20 mM HEPES, pH 7.5, 2M NaCl. The bound protein was eluted with a 1250 ml linear gradient to 20 mM HEPES, pH 7.5, 20% ethanol at a flow rate of 40 ml/minute. 25 ml fractions were collected. A major peak eluted at approximately 17.5% ethanol, as well as a minor peak at 13-15% ethanol. Aliquots (50μl) of the fractions were diluted to 200μl with Buffer C (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom), and eluted with 80% acetonitrile, 0.042% TFA (Buffer D) using a linear gradient (increase of 1% Buffer D/minute) at a flow rate of 0.1

ml/minute. The major peak eluting at 17.5% ethanol contained homogeneous, correctly refolded met-IGF-1. Fractions containing this peak were pooled, concentrated to 2 mg/ml, dialyzed against 100 mM HEPES, 44 mM sodium phosphate, pH 6.0, and stored at -70°C.

Relatively small quantities (50-100 μg) of pure, correctly folded recombinant met-IGF-1 could be obtained by injecting 75-150 mg of S-Sepharose pool B (in 1 ml of 0.05% TFA, Buffer C) onto a reverse phase column (RP-4, 4.6 x 250 mm) and eluting with 80% acetonitrile in water, 0.042% TFA (Buffer D) using a linear gradient (an increase of 1% Buffer D/min) at a flow rate of 0.5 ml/min. Correctly refolded met-IGF-1 eluted with a retention time of 58.2 min, was neutralized with 1 M Tris, pH 7 (0.15 ml/min), dialyzed against either 100 mM HEPES, 44 mM sodium phosphate, pH 6 (IGF binding buffer), or phosphate buffered saline (PBS) and stored at -70°C.

**EXAMPLE 4**

Conversion of Met-IGF-1 to IGF-1

In order to convert recombinant met-IGF-1 to native human IGF-1, an aminopeptidase, isolated from Aeromonas proteolytica using a modification of a previously described method (Lorand, L., 1976, Meth. Enzymol. 15: 53-543, incorporated herein by reference) was used to remove the N-terminal methionine. Recombinant met-IGF-1 was incubated in the presence or absence of the purified aminopeptidase in a 100 μl reaction mixture containing 120 μg met-IGF-1, 20 mM Tricine, pH 8.0, 1 μg aminopeptidase for 30 minutes at 25° C. The reaction was stopped by the addition of 1 ml 0.05% TFA in water. Aliquots of the samples were analyzed on a reverse phase column, and the protein peaks collected and subjected to sequence analysis. Met-IGF-1 eluted at 58.2 minutes; whereas, the material reacted with the aminopeptidase comigrated with natural human IGF-1 (Bachem, Torrence, CA) at 56 minutes. The following is a summary of the pmoles of each residue recovered at each sequence cycle, normalized for 100 pmoles of starting material:

**TABLE 1**

met-IGF-1 + 1 μg Aminopeptidase

| | Pmoles Recovered | | | | |
|---|---|---|---|---|---|
| Cycle | Met | Gly | Pro | Glu | Thr |
| 1 | 2.32 | 86.8 | 2.4 | 1.78 | 1.45 |
| 2 | 0.00 | 23.3 | 105.6 | 3.1 | 0.8 |
| 3 | 0.18 | 13.8 | 23.4 | 128.6 | 1.4 |
| 4 | 0.00 | 9.3 | 4.6 | 24.1 | 51.5 |

Sequence obtained: Gly, Pro, Glu, Thr; approximately 2% of the molecules did not have N-terminal Met cleaved by aminopeptidase.

**TABLE 2**

met-IGF-1 No Aminopeptidase

| | Pmoles Recovered | | | | |
|---|---|---|---|---|---|
| Cycle | Met | Gly | Pro | Glu | Thr |
| 1 | 88.16 | 11.6 | 2.15 | 1.62 | 0.0 |
| 2 | 4.8 | 108.4 | 2.31 | 4.1 | 2.0 |
| 3 | 0.5 | 25.8 | 80.72 | 7.6 | 1.3 |
| 4 | 0.0 | 15.9 | 25.2 | 71.6 | 0.9 |

Sequence obtained: Met, Gly, Pro, Glu

These results show that approximately 98% of met-IGF-1 was converted to native IGF-1 by the aminopeptidase.

**EXAMPLE 5**

Purification & Refolding of IGFBP-1

*E. coli* cells expressing the IGFBP-1 were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM DTT) at a concentration of 40 ml/10 g cell paste, and were disrupted at 1800 psi using a French pressure cell. The suspension was centrifuged 20,000 x g for 30 minutes, and aliquots of the pellet & supernatant were analyzed by SDS-PAGE. A major band corresponding to the IGFBP-1 was present in the pellet, but not the supernatant. The pellet was suspended in Buffer A (40 ml/10 g cell paste), and re-centrifuged at 20,000 x g for 30 minutes. This wash procedure was repeated 2 times. The final pellet containing the IGFBP-1 was suspended in 6M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT (25 ml/10 g cells) using a ground glass homogenizer. The suspension was incubated at room temperature for 15 minutes. The undissolved protein was removed by

centrifugation at 20,000 x g for 30 minutes. Final concentration of the IGFBP-1 was 1.0 mg/ml. SDS-PAGE analysis of the pellet and supernatant showed that IGFBP-1 was present in the supernatant only.

The denatured and reduced IGFBP-1 was subjected to a three-step refolding procedure.

a) Oxidized glutathione, the mixed-disulfide producing reagent (GSSG), was added to the supernatant to a final concentration of 25 mM, and incubated at room temperature for 15 minutes.

b) The solution was then diluted 10 fold gradually with 50 mM tris. pH 9.7 and phenylmethylsulfonylfluoride was added to final concentration of 1mM. Final concentration of protein was 100μg/ml.

c) The refolding mixture was incubated overnight at 4°C, and then centrifuged at 20,000 x g for 15 minutes. SDS-PAGE analysis of the pellet and supernatant showed that the supernatant was composed of relatively homogeneous IGFBP-1.

Aliquots (50μl) of the supernatant were diluted to 200μl with Buffer C (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom), and eluted with 80% acetonitrile, 0.042% TFA (Buffer D) using a linear gradient (increase of 1% Bufer D/minute) at a flow rate of 0.1 ml/minute.

A single major peak representing refolded IGFBP-1 eluted at 68 minutes. The retention time of the refolded IGFBP-1 shifted to 71.0 minutes after being completely reduced and denatured in 5 M guanidine, 50 mM Tris pH 7.5, 100 mM DTT. These results indicate that IGFBP-1 refolds to a single predominant species under the conditions described. N-terminal sequence analysis of IGFBP-1 eluting at 68.0 minutes gave the sequence MetAlaProTrpAsnCysAlaPro... (three letter amino acid code)(SEQ ID NO:13), which matches the N-terminal amino acid sequence of human IGFBP-1 except for an extra methionine residue at the N-terminus of the recombinant protein.

Isolation of Refolded IGFBP-1

The refold mixture (15000 ml) prepared from 590g of *E. coli* paste containing the correctly refolded IGFBP-1 was concentrated to 1800 ml, dialyzed against 20 mM sodium phosphate, pH 6.0,

centrifuged at 10,000 x g for 30 minutes to remove precipitated *E. coli* proteins and loaded onto an Q-Sepharose (Pharmacia/LKB, Piscataway, NJ) column (5.0 x 60 cm) previously equilibrated with the same buffer. The bound protein was eluted with a 5000 ml linear gradient to 0.5M NaCl at a flow rate of 20 ml/minute. 25 ml fractions were collected. A single major peak eluted at 0.3-0.4 M NaCl; 100 µl aliquots of each fraction were analyzed separately by a reverse phase chromatography column (RP-4 1 x 250 mm Synchrom). Fractions containing predominantly correctly refolded IGFBP-1 (determined from RP-4 analysis), were pooled (900 ml), the pH was adjusted to 7.5, the conductivity was adjusted to 1 mM NaCl (95 mOhm), and loaded onto a Toyopearl butyl-650 S hydrophobic interaction column (5 x 5 cm) (Supelco, Bellefonte, PA), previously equilibrated with 20 mm HEPES, pH 7.5, 1.0 M NaCl at a flow rate of 30 ml/minute.

The protein was eluted with a 1500 ml linear gradient to 20 mM HEPES, pH 7.5 at a flow rate of 40 ml/minute. A single broad peak eluted at 5 - 15% ethanol. Aliquots (10µl) of each peak fraction were analyzed by RP-4 reverse phase chromatography and SDS-PAGE. Fractions containing pure (95%) correctly refolded IGFBP-1 were pooled, concentrated to 6-8 mg/ml and assayed for bioactivity.

**EXAMPLE 6**

Activity of IGF-1 and IGFBP-1 on Mouse 3T3 Fibroblasts

A crystal violet dye assay was used to measure cell proliferation. Assays were performed in 96 well gelatin-coated plates. Balb/c 3T3 fibroblasts were plated at 25,000 cells/well in 200 µl of serum-free DMEM (Dulbecco's modification of Eagle's media, Mediatech, Herndon, VA) containing 0.03 M Glycerol and 0-1000 ng/ml IGF-1. Cells were incubated for 72 hours at 37°C . At this time, the media was replaced with 150 µl of 0.2% crystal violet, 10% formaldehyde, 10 mM potassium phosphate pH 7.0. After a 20 minute incubation at room temperature, the wells were washed 3 times with PBS, and the cell-bound dye released by incubation with 200 µl/well of 50% ethanol/0.1M sodium citrate, pH 4.2. Absorbance at 570 nm was read the next day. Results showed that recombinant IGF-1 stimulates proliferation of 3T3 fibroblast cells in a dose dependent manner. Maximal

proliferation occurred at a IGF-1 concentration of about 200 ng/ml. The $ED_{50}$ was approximately 20-30 ng/ml.

The effect of IGFBP-1 on IGF-1-stimulated proliferation of 3T3 fibroblasts was determined by co-incubating the cells with a set amount of IGF-1 and increasing amounts of IGFBP-1. Balb/c 3T3 fibroblasts were plated at 25,000 cells/well in 200 µl of serum-free DMEM containing 0.03 M glycerol and either 20ng or 50ng/ml IGF-1, and varying amounts of IGFBP-1 (100 ng/ml - 1 x $10^4$ ng/ml). The cells were incubated for an additional 72 hours and processed as described above. At both concentrations of IGF-1, co-incubation with a 5-fold molar excess of IGFBP-1 inhibited the proliferation of the fibroblasts by about 50%; a 10-15 fold molar excess of IGFBP-1 was sufficient to completely inhibit the IGF-1 dependent proliferation.

Activity of IGF-1 on Rat Osteosarcoma Cells

The mitogenic activity of IGF-1 was determined by measuring the relative amount of $^3$H-thymidine incorporated into DNA of rat osteosarcoma cells when varying amounts of IGF-1 were incubated with these cells under serum free conditions. Rat osteosarcoma cells (UMR106 cell line, American Type Culture Collection, Accession No CRL-1661, Rockville, Maryland) were plated at 5-6 x $10^4$ cells in 0.5 ml of Ham's F12 (Mediatech, Herndon, VA) containing 7% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates (Costar, Cambridge, MA). After incubating the plates for 72 hours at 37°C (at which time the cells were confluent, i.e., touching each other), the cells were washed twice with phosphate buffered saline (PBS) and pre-incubated an additional 24 hours in serum-free Ham's F12 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. After this pre-incubation in serum-free media, 0.5 ml of F12 serum-free media containing serial dilutions (1.0 - 1,000 ng/ml) of IGF-1 were incubated with the cells for an additional 20-24 hours. Each well was pulse-labeled with 0.5 µCi of $^3$H-thymidine (Cat. #NET-027Z, NEN Research Products, Du Pont Co., Boston, MA) for 4 hours, then washed with cold PBS three times. Incorporated $^3$H-thymidine was precipitated with cold 7% trichloroacetic acid (Cat. #0414-01, J.T. Baker Inc., Phillipsburg, NJ) and

quantitated by liquid scintillation counting. Assays were performed in triplicate.

These results showed that recombinant IGF-1 stimulates the proliferation of UMR106 rat osteosarcoma cells in a dose dependent manner. Maximal proliferation occurred at 100 - 200 μg/ml. The $ED_{50}$ was approximately 20 - 30 ng/ml.

The effect of recombinant IGFBP-1 on the IGF-1-stimulated mitogenesis of rat UMR106 osteosarcoma cells was determined using the assay described above. After the 24 hour pre-incubation with serum-free media, the cells were incubated for an additional 20-24 hours with 50 ng/ml IGF-1 plus different amounts of recombinant IGFBP-1, ranging from 100 ng/ml to 10,000 ng/ml. We found that at a molar ratio of 20:1 (IGFBP-1:IGF-1), IGFBP-1 reduced the mitogenic activity of IGF-1 by 80%.

These results also showed that IGF-1 is biologically active when tested in vitro using two different types of cells, murine Balb-C 3T3 fibroblasts and UMR106 rat osteosarcoma cells. Furthermore, IGFBP-1 inhibited IGF-1 dependent proliferation of these cells demonstrating its ability to bind IGF-1 and prevent the binding of IGF-1 to cell surface IGF receptors. In both of these assays a molar ratio of 10-20:1 (IGFBP-1:IGF-1) inhibited the biological activity of IGF-1 by 80-100%.

## EXAMPLE 7

### DIABETIC (db/db) MOUSE MODEL

Homozygous db/db mice are diabetic and exhibit delayed wound-healing compared to normal mice or heterozygous db/+ mice (Tsuboi and Rifkin, Journal of Experimental Medicine, 172:245-251, 1990). The following experiments were designed to compare the effects of IGF-1 and IGFBP-1 on wound-healing in this model. The IGF-1 used in these experiments is met-IGF-1.

Female db/db mice (approximately 8 weeks of age) were randomly assigned to treatment groups. Each mouse received two 6 mm full-thickness circular wounds on the center of the back using a 6 mm punch biopsy instrument. Growth factors or control solution, phosphate buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA (Sigma Chemical Company, Chicago, IL) were applied to the wounds in 20 μl. Solutions were applied daily for 5 days. Wounds were left open during this time.

Animals were sacrificed on day 8 and histological sections of the wounds prepared at their widest margin. Parameters measured were: (1) percent re-epithelialization; (2) new granulation tissue; and (3) capillary number.

Re-epithelialization was determined by calculating the ratio of the length of re-epithelialized epidermis versus the original wound width using tracings of the histological sections on a TV monitor at 40 X magnification. Data are expressed as percentages.

New granulation tissue was determined from tracing the margins of the granulation tissue in each wound on a TV monitor. Data are expressed as an area ($mm^2$).

Capillary number was determined by counting the number of capillary lumens in whole wound cross sections of the granulation tissue at 100 x magnification.

Experiment 1

The first experiment was designed to compare increasing amounts of an approximate 1:1 molar ratio of IGF-1:IGFBP-1 to saline controls. The highest dose tested a 1:0.5 molar ratio of the two proteins. The four treatment groups were as follows:

1. control 5 mice
2. 1 μg IGF-1 + 4 μg IGFBP-1 4 mice
3. 10 μg IGF-1 + 40 μg IGFBP-1 4 mice
4. 50 μg IGF-1 + 100 μg IGFBP-1 4 mice

The results of this experiment are shown in Table 3.

TABLE 3

EFFECTS OF INCREASING CONCENTRATIONS OF THE COMBINATION OF IGF-1 PLUS IGFBP-1 ON WOUND-HEALING PARAMETERS IN DIABETIC (db/db) MICE

| Treatment Group | Reepithelialization | | New Granulation Tissue | | Capillaries | |
|---|---|---|---|---|---|---|
| | Percent | Percent of Control | $mm^2$ | Percent of Control | Number | Percent of Control |
| | | | | | | |
| Control | 41.0±8.3 | 100% | 3.88±0.23 | 100% | 45.0±4.5 | 100% |
| IGF-1 (1 μg) + IGFBP-1 (4 μg) | 48.9±8.1 | 119% | 3.71±0.29 | 96% | 53.0±9.3 | 118% |
| IGF-1 (10 μg) + IGFBP-1 (40 μg) | 53.9±7.6 | 131% | 4.19±0.31 | 108% | 64.0±8.5 | 142% |
| IGF-1 (50 μg) + IGFBP-1 (100 μg) | 63.8±9.4 | 156% | 4.35+0.53 | 112% | 63.1±6.0 | 140% |

The combination of IGF-1 + IGFBP-1 caused a dose-dependent increase in the rate of re-epithelialization and in capillary number (+ 156% and + 140%, respectively, compared to controls, at the highest dose). There was a less significant increase in granulation tissue (+112%).

Experiment 2

This experiment was designed to determine whether the positive effects seen in experiment 1 were due to IGF-1, IGFBP-1, or the combination of IGF-1 + IGFBP-1.

Treatment groups were:

| | |
|---|---|
| 1. control | 9 mice |
| 2. 50 μg IGF-1 | 8 mice |
| 3. 165 μg IGFBP-1 | 8 mice |
| 4. 50 μg IGF-1 + 165 μg IGFBP-1 | 8 mice |

The results of this experiment are shown in Table 4. The positive effects of the combination of IGF-1 + IGFBP-1 on re-epithelialization and capillary number were repeated (+133% and +143%, respectively, compared to controls). A better response of the two factors in stimulating granulation tissue was seen in this experiment (+161%, compared to controls). The combination of IGF-1 + IGFBP-1 was better than either protein alone.

TABLE 4

EFFECTS OF IGF-1, IGFBP-1 OR THE COMBINATION OF IGF-1 + IGFBP-1
ON WOUND-HEALING PARAMETERS
IN DIABETIC (db/db) MICE

| Treatment Group | Reepithelialization | | New Granulation Tissue | | Capillaries | |
|---|---|---|---|---|---|---|
| | Percent | Percent of Control | $mm^2$ | Percent of Control | Number | Percent of Control |
| | | | | | | |
| Control | 48.3±6.4 | 100% | 2.41±0.19 | 100% | 43.2±2.3 | 100% |
| IGFBP-1(165μg) | 53.4±5.5 | 111% | 2.47±0.20 | 123% | 52.9±4.7 | 122% |
| IGF-1 (50 μg) | 52.9±6.1 | 110% | 3.10±0.24 | 129% | 57.5±3.6 | 133% |
| IGF-1 (50 μg) + IGFBP-1(165 μg) | 64.0±6.0 | 133% | 3.89+0.24 | 161% | 61.8±4.9 | 143% |

## EXAMPLE 8

### RABBIT EAR DERMAL ULCER MODEL

This experiment was designed to test the effects of IGF-1 and IGFBP-1 in the rabbit ear full thickness dermal ulcer model (Mustoe et al., Journal of Clinical Investigation, 87:694-703, 1991). This wound healing model precludes significant wound contraction and requires new granulation tissue and epithelial cells for healing to originate centripically. The effects of IGF-1 alone, IGFBP-1 alone and combinations of the two factors in three different molar ratios were compared.

Twenty four young adult New Zealand white rabbits (3.0-3.5 kg) were divided into six comparison groups. Surgical procedures were performed under an operating microscope and under sterile conditions. Rabbits were anesthetized by an intramuscular injection of a mixture of ketamine (60 mg/kg) and zylazine (5 mg/kg). The hair was clipped from the inside and along the margins of both ears. Each ear was immobilized and four 6 mm ulcers were made to the depth of bare cartilage using a 6 mm trephine. This wounding procedure leaves bare cartilage with the perichondrium removed. Growth factors or vehicle solution were applied once only to the wounds at the time of wounding in a volume of 5 $\mu$l. The wounds were then covered with an occlusive dressing (Tegaderm, 3M Corporation, Minneapolis, MN). The left and right ears of the rabbits in the different comparison groups received the growth factor or control solutions indicated in Table 5.

TABLE 5

TESTING PROTOCOL FOR IGFBP-1 AND IGF-1
IN THE RABBIT DERMAL ULCER MODEL

| TREATMENT GROUP | LEFT EAR | RIGHT EAR | IGF1:IGFBP-1 MOLAR RATIO |
|---|---|---|---|
| Group 1 | Buffer | IGF-1 1 μg/IGFBP-1 0.3 μg | 11:1 |
| Group 2 | IGF-1 5 μg | IGF-1 5 μg/IGFBP-1 1.5 μg | 11:1 |
| Group 3 | IGFBP-1 13.2 μg | IGF-1 4 μg/IGFBP-1 13.2 μg | 1:1 |
| Group 4 | Buffer | IGF-1 1 μg/IGFBP-1 3.3 μg | 1:1 |
| Group 5 | IGF-1 5 μg | IGF-1 5 μg/IGFBP-1 3 μg | 5:1 |
| Group 6 | IGFBP-1 9 μg | IGF-1 15 μg/IGFBP-1 9 μg | 5:1 |

Rabbits were sacrificed 7 days post-wounding. Each wound was bisected, fixed in formalin and 5 μm cross sections taken for histological analysis. Measurements were made under a light microscope using a lens micrometer. Parameters measured were: new granulation tissue (horizontal migration of new granulation tissue measured from the original wound edge); granulation tissue gap (distance between the advancing edges of the new granulation tissue), epithelial gap (distance between the advancing edges of the new epithelium) and superficial peak height (vertical distance between the cartilage and the maximum height of the new granulation tissue).

The results of this experiment are presented in Tables 6 and 7. The combination of IGF-1 + IGFBP-1 significantly stimulated new granulation tissue formation when IGF-1 was applied in molar excess (the 5:1 and 11:1 IGF-1:IGFBP-1 groups). The effects seen were dose-dependent. The equimolar combinations of IGF-1 + IGFBP-1 gave variable responses, with the low dose group showing

stimulation and the high dose group showing no stimulation above control values. The effects of the combination of IGF-1 + IGFBP-1 on re-epithelialization and height of new granulation tissue were less striking. The latter two parameters are less reliable indicators in this model.

TABLE 6

EFFECTS OF IGF-1, BP-1 AND THE COMBINATION OF IGF-1 AND BP-1 ON WOUND HEALING PARAMETERS IN THE RABBIT DERMAL ULCER MODEL

| TREATMENT GROUP | TREATMENT | NEW GRANULATION TISSUE (mm) | GRANULATION TISSUE GAP (mm) | SUPERFICIAL PEAK HEIGHT (mm) | EPITHELIAL GAP (mm) |
|---|---|---|---|---|---|
| Group 1A | Buffer | 0.859±0.125 | 4.388±0.118 | .503±0.017 | 3.525±.203 |
| Group 1B | IGF-1 1μg/ IGFBP-1 0.3μg | 1.256±0.138 | 4.091±0.101 | .503±0.018 | 2.563±.296 |
| Group 2A | IGF-1 5μg | 0.900±0.144 | 4.650±0.156 | .488±0.013 | 2.591±.501 |
| Group 2B | IGF-1 5μg/ IGFBP-1 1.5μg | 1.413±0.114 | 4.094±0.144 | .603±0.047 | 2.094±.347 |
| Group 3A | IGFBP-1 13.μg | 0.784±0.111 | 4.756±0.174 | .503±0.031 | 2.366±.409 |
| Group 3B | IGF-1 4μg/ IGFBP-1 13.2μg | 0.791±0.125 | 4.678±0.175 | .509±0.018 | 2.481±.346 |
| Group 4A | Buffer | 0.972±0.960 | 4.638±0.122 | .472±0.015 | 1.888±.314 |

TABLE 6 (Continued)

| TREATMENT GROUP | TREATMENT | NEW GRANULATION TISSUE (mm) | GRANULATION TISSUE GAP (mm) | SUPERFICIAL PEAK HEIGHT (mm) | EPITHELIAL GAP (mm) |
|---|---|---|---|---|---|
| Group 4B | IGF-1 1μg/ IGFBP-1 3.3μg | 1.391±0.113 | 4.419±0.157 | .509±0.0221 | 1.538±.347 |
| Group 5A | IGF-1 5μg | 0.919±0.139 | 4.444±0.162 | .478±0.016 | .941±.298 |
| Group 5B | IGF-1 5μg/ IGFBP-1 3μg | 1.384±0.115 | 3.978±0.138 | .534±0.027 | 1.266±.287 |
| Group 6A | IGFBP-1 9μg | 0.775±0.101 | 4.60±0.140 | .466±0.013 | 2.347±.310 |
| Group 6B | IGF-1 15μg/ IGFBP-1 9μg | 1.488±0.104 | 4.088±0.104 | .506±0.019 | .847±.248 |

TABLE 7

NEW GRANULATION TISSUE STIMULATED
BY THE COMBINATION OF IGF-1 AND IGFBP-1 VERSUS
IGF-1 ALONE, BP-1 ALONE OR BUFFER*

| | PERCENT OF COMPARISON GROUP | | | | | |
|---|---|---|---|---|---|---|
| TREATMENT GROUP | BUFFER GROUP 1A | BUFFER GROUP 4A | IGF-1 GROUP 2A | IGF-1 GROUP 5A | IGFBP-1 GROUP 3A | IGFBP-1 GROUP 6A |
| 1B. IGF-1 1µg/ IGFBP-1 0.3 µg (11:1) Molar Ratio | + 46% | + 29% | + 40% | + 37% | + 60% | + 62% |
| 2B. IGF-1 5µg/ IGFBP-1 1.5µg (11:1) Molar Ratio | + 64% | + 45% | + 57% | + 54% | + 86% | + 82% |
| 3B. IGF-1 4µg/ IGFBP-1 13.2µg (1:1) Molar Ratio | - 8% | - 19% | - 12% | - 14% | + 1% | + 2% |
| 4B. IGF-1 1µg/ IGFBP-1 3.3µg (1:1) Molar Ratio | + 62% | + 43% | + 55% | + 51% | + 77% | + 79% |

TABLE 7 (Continued)

| | PERCENT OF COMPARISON GROUP | | | | | |
|---|---|---|---|---|---|---|
| TREATMENT GROUP | BUFFER GROUP 1A | BUFFER GROUP 4A | IGF-1 GROUP 2A | IGF-1 GROUP 5A | IGFBP-1 GROUP 3A | IGFBP-1 GROUP 6A |
| 5B. IGF-1 5μg/ IGFBP-1 3μg (5:1) Molar Ratio | + 61% | + 42% | + 54% | + 51% | + 77% | + 79% |
| 6B. IGF-1 15μg/ IGFBP-1 9μg (5:1) Molar Ratio | + 73% | + 53% | + 65% | + 62% | + 90% | + 92% |

Numbers shown are the percent increase or decrease in new granulation tissue in treatment groups 1B-6B relative to comparison groups 1A-6A. Data from Table 6 were used for these calculations.

IGF-1 alone or IGFBP-1 alone were not effective in stimulating new granulation tissue formation above the levels seen with buffer solution.

## EXAMPLE 9

### RABBIT EAR DERMAL ULCER MODEL (ISCHEMIC)

Young adult female New Zealand white rabbits (3.0-4.0 kg) were divided into 2 groups. In one group, four 6mm dermal ulcers were made in normal ears as described in Mustoe et al., J. Clinical Invest., 87:694-703 (1987) and above in Example 8.

In the second group, three 6mm ulcers were made in ischemic ears as described in Ahn and Mustoe, Ann. Plastic Surgery, 24:17-23 (1990). Ischemic ears were rendered in 28 (56 ears) rabbits. The ears were reproducibly ischemic with a tissue $pO_2$ measured with a tissue optode of 20-28. A full thickness circumferential incision was made around the base of the rabbit ears, sparing the cartilage, the three major veins and the caudal artery. All of the minor veins, the arterioles, the ventral artery and the central artery were divided and the tissues cut. The skin was sutured by continuous method. The wounds were made in an inverted L-pattern, with the long arm over the ventral artery and the base over the central artery, avoiding the area near the caudal artery. The wounds extended to bare cartilage and the central tissue was removed. Each animal was examined daily for infection, disruption of the wound, or loss or displacement of the wound covering. In the ischemic model, the wounds were made immediately following the creation of the ischemia. Wound healing was profoundly impaired. This model is a major improvement over previously known wound healing models in reproducing the hypoxic (ischemic) environment that exists in many human chronic wounds.

IGF-1 and/or IGF-BP1 in varying ratios and varying concentration or control buffer were applied at the time of wounding, and occlusive dressings were applied. Wounds were harvested at day 7 for histologic analysis.

Test substances were applied to the wounds once at the time of wounding as described in Example 8. Following administration of the test growth factors, the wounds were covered with an occlusive polyurethane film (TEGADERM™, 3M Company, Minneapolis, MN). At day 7, the rabbits were anesthetized. The entire ear was then removed and fixed in 10% buffered neutral formaldehyde overnight or in omnifix for 2.5 hours. The wound was partially excised by taking a through and through rectangular block, including both skin surfaces and the cartilage. The animals were then sacrificed.

In the histological analysis, the tissues were dehydrated in graded alcohol and xylene and then embedded in paraffin according to standard procedures. The wounds were bisected and sectioned at 6 μm to obtain a cross-section near the center of the wound. The section was mounted on poly-L-lysine coated slides and stained with hematoxylin and eosin. The slides were observed under an ordinary light microscope.

Measurements of various parameters indicative of the rate of healing were then taken. The parameters measured included: new epithelial growth ("N-EG"); the distance between advancing edges of granulation tissue ("P-P" also referred to as granulation tissue gap in Example 8); the height of new granulation tissue ("SP" also referred to as the superficial peak height in Example 8), new granulation tissue ("TNG"); and volume of new wound healing tissue ("N-volume"). N-volume was calculated using the assumption wounds heal concentrically according to the standard volume formula measured from the difference between the volume of wound at day 0 and the volume at day 7, the time of sacrifice. P-P is a direct measurement of the horizontal distance between migrating granulation tissue edges from histologic cuttings. For P-P measurements, a smaller number indicates more effective healing. TNG is a measurement of new granulation tissue growth based on differences in staining in new collagen versus the mature collagen of intact dermis. N-EG is a measurement of the

horizontal migration of new epithelial tissue measured from the original wound edge.

Tables 8 and 9 provide a summary of the results obtained in the non-ischemic model of Example 8 and the ischemic model described above, respectively. All amounts of proteins indicated in Tables 8 and 9 are in μg doses.

The results shown in Table 8 are consistent with those obtained in the non-ischemic studies described in Example 8. The results indicate that a molar excess of IGF-1 over IGF-BP1 is preferred, while neither alone are as effective. In addition, there appears to be a dose dependent effect. When used in molar ratios of 5:1 to 11:1, an increase in new granulation tissue formation and epithelialization was observed, with the ratio of 10:1 (43.2 μg IGF-1 to 12.9 μg IGF-BP1) providing the maximal effect, within the dose range tested. In the ischemia model, the 43.2 μg/12.9 μg (10:1) ratio proved to be highly effective.

TABLE 8

NON-ISCHEMIC MODEL

| CONDITION | N-EG[1] | P-P[1] | SP[1] | TNG[1] | N-VOLUME | NUMBER OF WOUNDS |
|---|---|---|---|---|---|---|
| BUFFER | 367 | 470 | 50.5 | 100 | 4.2 E+06 | 65 |
| IGF (5 μg) | 413 | 455 | 48 | 91 | 3.5 E+06 | 32 |
| IGF (43.2) | 355 | 440 | 49 | 93 | 4.0 E+06 | 12 |
| BP1 (9) | 364 | 452 | 46 | 100 | 3.7 E+06 | 28 |
| BP1 (13.2) | 353 | 476 | 50.3 | 78 | 3.4 E+06 | 16 |
| IGF1/BP1(1:1): | | | | | | |
| 1/3.3 | 440 | 472 | 50 | 114 | 4.6 E+06 | 27 |
| 4/13.2 | 342 | 468 | 51 | 79 | 3.4 E+06 | 16 |
| IGF1/BP1(5:1): | | | | | | |
| 3/5 | 463 | 398 | 53.4 | 138 | 5.8 E+06 | 16 |
| 15/9 | 505 | 409 | 50.6 | 149 | 5.9 E+06 | 16 |
| IGF/BP1(11:1): | | | | | | |
| 1/0.3 | 334 | 409 | 50.3 | 126 | 5.1 E+06 | 16 |
| 5/1.5 | 381 | 413 | 60 | 141 | 6.9 E+06 | 16 |
| IGF1/BP1(10:1) | | | | | | |
| 43.2/12.9 | 497 | 345 | 52 | 219 | 8.1 E+06 | 12 |

[1] measured in mm x $10^2$

TABLE 9

ISCHEMIC MODEL

| CONDITION | N-EG | P-P | SP | TNG | N-VOLUME | NUMBER OF WOUNDS |
|---|---|---|---|---|---|---|
| IGF/BP1 (5:1): | | | | | | |
| 15/9 | 133 | 546 | 40.3 | 32 | 1.15 E+06 | 11 |
| BUFFER | 121 | 539 | 40.5 | 28 | 1.05 E+06 | 12 |
| P-VALUE | *NS | *NS | *NS | *NS | *NS | |
| IGF1/BPA(10:1): | | | | | | |
| 43.2/12.9 | 278 | 519 | 42.7 | 64.7 | 2.26 E+06 | 6 |
| BUFFER (2 RABBITS) | 102 | 557 | 40.7 | 22 | 8.00 E+05 | 6 |
| P-VALUE | 0.055 | *NS | *NS | <0.05 | <0.03 | |
| IGF/BP1(10:1): | | | | | | |
| 43.2/12.9 | 468 | 460 | 42.8 | 101.7 | 3.60 E+06 | 12 |
| BUFFER (4 RABBITS) | 259.5 | 543 | 42.5 | 36 | 1/23 E+06 | 8 |
| P-VALUE | <0.01 | <0.05 | *NS | <0.01 | <0.0001 | |

*NS=not significant, p>0.05

The statistical analysis was carried out using Student's paired t-test for each group studied. All values are expressed as mean +/- SEM. A p- value of 0.05 or less was considered significant.

Although this invention has been described with respect to specific embodiments, it is not intended to be limited thereto and modifications made by those skilled in the art are considered to fall within the spirit and scope of the instant invention.

We claim:

1. A pharmaceutical composition for wound healing comprising therapeutically effective amounts of IGF-1 or active fragments thereof and IGFBP or active fragments thereof in a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the IGFBP is IGFBP-1.

3. The pharmaceutical composition of claim 2, wherein the ratio of IGF-1 to IGFBP-1 is up to 100:1.

4. The pharmaceutical composition of claim 3, wherein the ratio of IGF-1 to IGFBP-1 is in the range of 1:1 to 11:1.

5. The pharmaceutical composition of claim 4, wherein the ratio of IGF-1 to IGFBP-1 is about 5:1.

6. The pharmaceutical composition of claim 4, wherein the ratio of IGF-1 to IGFBP-1 is about 10:1.

7. The pharmaceutical composition of claim 2, wherein the pharmaceutical composition is a liquid, cream, lotion, gel, aerosol, powder, or solid.

8. The pharmaceutical composition of claim 2, wherein the pharmaceutical composition is a liquid.

9. A method for using the pharmaceutical composition of claim 1 to treat wounds comprising administering the pharmaceutical composition non-systemically to a patient in need thereof.

10. The method of claim 9, wherein the IGFBP is IBFBP-1.

11. The method of claim 10, wherein the IGFBP is IGFBP-1.

12. The method of claim 10, wherein the pharmaceutical composition is administered topically, intradermally, intrarectally, orally, or by inhalation.

13. The method of claim 10, wherein the pharmaceutical composition is administered topically.

14. The method of claim 10, wherein said wound is dermal.

15. The method of claim 10, wherein said wound is a burn, surgical wound, traumatic interruption of connective tissue, disruption of mucous membrane, corneal trauma, or transplant.

16. The method of claim 10, wherein the ratio of IGF-1 to IGFBP-1 is about 5:1.

17. The method of claim 10, wherein the ratio of IGF-1 to IGFBP-1 is 1:1 to 11:1.

18. The method of claim 10, wherein the ratio of IGF-1 to IGFBP-1 is about 10:1.